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(54) Title: DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATION AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

(57) Abstract

The invention relates to a method for detecting specific target-cells in a simple and time saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. The method can further be used for isolation of the target-cells by magnetic field application and a kit for performing the method according to the invention is described.

Detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations.

The present invention relates to an immunomagnetic method for detection and positive isolation of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit for performing the method in different cell populations.

In biology, biochemistry and adjacent fields it is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sofisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sofisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen- antibody, enzyme- receptor, ligand- receptor interactions on cells and biotin- avidin binding, of which antigen-antibody binding is most frequently used. A hapten/anti-hapten binding pair method has also recently been suggested (PCT/EP90/01171).

When such methods are used for isolation of specific cells, which are the subject for further various examinations, it is necessary to reverse the linkage without producing destructive effects on the binding partners, which ideally should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for

adequately cleaving antigen/anti-antigen and hapten/anti-hapten linkages (PCT/EP91/00671, PCT/EP90/01171).

Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles or beads, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/-antibody/particle-complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (PCT/EP90/01171) and also a method for isolating haemopoietic progenitor cells from bone marrow (PCT/EP90/02327). The latter is directed to use a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haemalopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles.

PCT/EP90/01171 relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, anti-hapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later

cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

There is a need for a simple linkage to connect a target cell to an insoluble support, which do not involve compounds of a rather unspecified haptene-group, and which is directed to detection of specific target cells and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

Thus the object of the present invention is to detect specific target cells. It represents a sensitive detection method for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and exeedingly simple. Furthermore, the present method can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. A further object of the invention is to provide a kit for performing the method as characterized in the claims.

The intensions of the inventions are obtained by the method and kit characterized in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cells populations is suitable both for detection, and in addition positive isolation of both normal cells and patogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as paramagnetic particles or beads, which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the

membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood.

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cytosentrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and

immunofluorescence. Thus, slides prepared from biopsied tumors or cytosentrifugates have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cellsuspension an use a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flowcytometric examiniations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence (Beiske et al., Am. J. Pathology 141 (3), September 1992). Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a high number of cells can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The monoclonal antibodies used bind specifically to, for example, tumor cells and not to other cells than the target-cells present in the blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC-31), Cluster 2 (MW~40kD) antigen (NrLu10) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insuline receptor, insuline-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells, in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other

disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibody-coated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25°C, preferably at 4°C, under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20°C, preferably 4°C, under gentle rotation. The paramagnetic particles or beads, uncoated or precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25°C, preferably 4°C under gentle agitation. Samples of the cell suspension are then transferred to a cell counting device, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The number of antibody-coated beads added to the cell suspension should be between 2-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells.

For specific purposes, and in the cases where the density of the target-cells is low for example malignant cells, or the target-cells represent a very low fraction of the total number of cells ($\leq$ 1%), the target cells can be positively separated

from non-target cells in a magnetic field. The isolated target cells, can then be enumerated microscopically and the fraction of target cells relative to the total number of cells in the initial cell suspension can be calculated. Moreover, the target-cells may be characterized for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity.

With the existing methods, signals obtained on Southern, Northern and Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerasse chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic

hydrogen bonding network may result in delocalization of the positive charge on the NH_2-terminal NH_3^+, and the removal of the hydroxyl group of either Tyr^7 or Tyr^{171} could therefore destabilize the complex by disrupting this network. Thus, an intact hydrogen bonding system may be essential to bind an NH_3^+ in this region. Additionally, the binding of NH_3^+ could signal a conformational change that would lock the peptide nonamer into the site, particularly since the tyrosines involved are derived from all major structural elements forming the cleft (Tyr^{59} on the α1 helix, Tyr^{171} on the α2 helix, and Tyr^7 on the floor of the cleft). In either case, the disruption of the hydrogen bonding system would result in a large change in binding affinity (measured indirectly in this case by the CTL titration value), as observed.

In the atomic model, the hydroxyls of Tyr^{84} and Thr^{143} are positioned to hydrogen bond to the terminal carboxylate group of a short peptide, and therefore presumably to the last carbonyl group in the binding site of a longer peptide (5). The failure of the mutations Y84F and T143V to affect CTL recognition (Fig. 3) is not an unexpected result of the removal of a single hydrogen bond and may suggest a role in the mutants for water molecules in replacing the hydrogen bonds to the negatively charged peptide carboxyl group. Approximately 20 hydrogen bonds as well as van der Waals contacts involving approximately 80 to 100 atoms in 20 to 30 MHC side chains serve to bind a peptide in the cleft of a class I MHC molecule (9). Thus, the loss of a single hydrogen bond should represent a negligible loss in binding energy. Lys^{146} also appears to interact with the terminal carboxylate. A major difference between pockets A and F is the presence in the F pocket of the terminal NH_3^+ of Lys^{146} to neutralize the terminal carboxylate, whereas the A pocket does not contain a carboxylate to neutralize the terminal NH_3^+ of the nonapeptide directly (although a long range salt bridge to Glu^{63} is mediated through Tyr^7 and a water molecule) (9). This difference may also contribute to the functional importance of the hydrogen bonding network in the A pocket.

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Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction

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Effective methods are needed to identify and isolate those genes that are differentially expressed in various cells or under altered conditions. This report describes a method to separate and clone individual messenger RNAs (mRNAs) by means of the polymerase chain reaction. The key element is to use a set of oligonucleotide primers, one being anchored to the polyadenylate tail of a subset of mRNAs, the other being short and arbitrary in sequence so that it anneals at different positions relative to the first primer. The mRNA subpopulations defined by these primer pairs were amplified after reverse transcription and resolved on a DNA sequencing gel. When multiple primer sets were used, reproducible patterns of amplified complementary DNA fragments were obtained that showed strong dependence on sequence specificity of either primer.

Higher organisms contain about 100,000 different genes, of which only a small fraction, perhaps 15%, are expressed in any individual cell. It is the choice of which genes are expressed that determines all life processes—development and differentiation (1), homeostasis, response to insults, cell cycle regulation (2, 3), aging, and even programmed cell death. The course of normal development as well as the pathological changes that arise in diseases such as cancer (4), whether caused by a single gene mutation or a complex of multigene effects, are driven by changes in gene expression. Altered gene expression lies at the heart of the regulatory mechanisms that control cell biology. Comparisons of gene expression in different cell types provide the underlying information we need to analyze the biological processes that control our lives.

Current methods to distinguish mRNAs in comparative studies rely largely on the subtractive hybridization technique (5). A fingerprinting technique for mRNAs by two-dimensional (2-D) electrophoresis, such as has been used extensively in detecting cellular protein species (6), would be very useful. Reproducibility should be sufficient so that side-by-side comparisons of the mRNAs from different cells are possible. Furthermore, the identified spots should be usable for identifying and isolating the corresponding genes, mRNAs, or cDNAs. When protein gels were used frustration often followed because of the inability to obtain enough of the identified proteins for molecular characterization (7).

Our method is directed toward the identification of differentially expressed genes among the approximately 15,000 individual mRNA species in a pair of mammalian cell populations (8), and then recovering their cDNA and genomic clones. The general strategy is to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction (PCR). These short sequences are then displayed on a sequencing gel. Pairs of primers are selected so that each will amplify DNA from about 50 to 100 mRNAs because this number is optimal for display on the gel.

Selection of 3′ primers takes advantage of the polyadenylate [poly(A)] tail present on most eukaryotic mRNAs (9) to anchor the primer at the 3′ end of the mRNA, plus two additional 3′ bases. A primer such as 5′-T_{11}CA would allow anchored annealing to mRNAs containing TG located just upstream of their poly(A) tails (10). By probability this primer will recognize one-twelfth of the total mRNA population because there are 12 different combinations of the last two 3′ bases, omitting T as the

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penultimate base. The primer permits initiation of reverse transcription of only this subpopulation.

Any reverse transcribed cDNA species would be amplified by PCR if the distance at which a second primer anneals is smaller than 2 to 3 kb from the beginning of the poly(A) tail (an average molecular size of mRNA is 1.2 kb). Ideally this annealing position should be within 500 bp because cDNAs up to 500 bp can be resolved by size on a DNA sequencing gel. For a 5′ primer of arbitrary base sequence, annealing positions to cDNAs should be randomly distributed in distance from poly(A) tail. Therefore, the amplified products from various mRNAs will differ in size. After these PCR products have been labeled with [α-^{35}S]-labeled deoxyadenosine triphosphate (dATP), they would be displayed by autoradiography as a ladder on a sequencing gel.

The 5′ primer should in theory be short, 6 to 7 bp, for it to anneal fairly frequently near the end of a cDNA strand (Table 1). A critical technical problem is whether such short primers can give specific DNA amplification by PCR. Although arbitrary primers 8 to 10 nucleotides in length have been used for DNA polymorphism analysis by PCR (*11*), the standard PCR method uses primers of 20 or more nucleotides in length. After numerous trials with different primer sets and PCR conditions with cloned murine thymidine kinase (TK) cDNA (*12*) as a model template, the PCR parameters were chosen such that 42°C annealing would be optimal for product yield and specificity, whereas a 30-s elongation time would allow amplification of short products that could be resolved by a DNA sequencing gel. Primer $T_{11}CA$ in combination with a 10-mer (Ltk3) was found to give specific DNA amplification under these conditions (Fig. 1A). The specificity of DNA amplification dramatically increased with decreasing deoxynucleoside triphosphate (dNTP) concentration from that of standard conditions. Lowering the dNTP concentration to 2 μM not only improved the specificity of DNA amplification but also was necessary for labeling PCR products to a high enough specific activity with [α-^{35}S]-labeled dATP to provide high resolution on a DNA sequencing gel (Fig. 1C).

Primer-dependent DNA amplification was demonstrated because both primers were necessary for the specific amplification of the 301-bp TK cDNA fragment when either purified plasmid or lysate of *Escherichia coli* containing the plasmid was used as template (Fig. 1, B and C). The absence of bands in the *E. coli* genomic background demonstrates specificity of the reaction. Thus, a successful specific DNA amplification was achieved with a short primer set in combination with high resolution of the DNA sequencing gel.

Next, the method was applied to detect a subset of mRNAs in mammalian cells by comparing the TK message from quiescent versus cycling mouse A31 cells. Total RNAs (Fig. 2A) or mRNAs (Fig. 2B) were reverse transcribed with $T_{11}CA$. ; primer followed by PCR in the presence of the Ltk3 5′ primer. On the DNA sequencing gel, 50 to 100 amplified mRNAs ranging from 100 to 500 bp were visible, a number that seems to be optimal for analysis. Patterns of mRNA species seen between cycling and quiescent A31 cells (Fig. 2A) were very similar as expected, though specific differences were apparent. A band corresponding in size to the expected TK mRNA fragment was seen in the cycling cells but not in the quiescent cells obtained after serum starvation (Fig. 2A), which is consistent with TK mRNA production being G1/S phase specific (*13*). This experiment demonstrated that scarce mRNA species such as that of TK with copy numbers around 30 per cell can be detected (*13*). Repeated experiments produced highly reproducible patterns of cDNAs (95% bands were reproducible for a given pair of primers and mRNA sample in more than three independent experiments) (*14*). The reproducibility allowed direct comparisons between lanes (Fig. 2), further indicating the reliability of this method.

When normal (A31) and tumorigenic (BPA31) cells were compared, most bands were the same, but a few bands such as N1 were seen only in normal cells, or T1 in tumor cells (Fig. 2B). The band corresponding in size to the amplified TK mRNA fragment was clearly visible in both types of cells, representing a good internal control.

In accord with predictions, patterns of amplified cDNAs from human breast cancer and normal mammary epithelial cells were totally changed when either primer was changed (Fig. 3). Examination of these patterns provides further information regarding the differential display technique. When cDNA species were amplified with primer sets that differed by either the arbitrary primer (compare Fig. 3, lanes 1 and 2 with 3 and 4 and 5 and 6; lanes 7 and 8 with

Table 1. Theoretical calculation and experimental data of the number of mRNA species that can be amplified by arbitrary primers with different lengths in combination with an anchored oligo(dT) primer that binds to one-twelfth of the mRNA 3′ termini. The theoretical calculation is based on the estimation that a mammalian cell expresses about 15,000 different mRNA species (*8*) and that only amplified cDNA fragments with sizes smaller than 500 bp are visualized by a DNA sequencing gel.

Length of arbitrary primer (bases)	Kilobases per binding site	mRNA displayed (no.)	
		Theory	Experimental
6	4	150	0
7	16	38	0
8	65	10	0
9	262	2	20–30
10	1049	<1	50–100

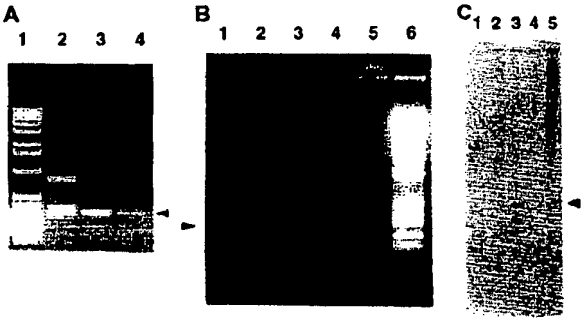

Fig. 1. Specific amplification of mouse TK cDNA template with primer set, $T_{11}CA$ and Ltk3 (CTTGATTGCC), which is located 278 bp upstream of its poly(A) tail (*12*). (**A**) A 1.5% agarose gel showing dNTP concentration dependence of the specificity of DNA amplification. The 10 ng of mouse TK cDNA plasmid pAMTK was amplified with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Connecticut) in the presence of 2.5 μM $T_{11}CA$, 0.5 μM Ltk3 with dNTP concentrations at 200 μM (lane 2), 20 μM (lane 3), and 2 μM (lane 4). Other components in the PCR reaction were as suggested by the manufacturer. PCR parameters were 94°C for 30 s, 42°C for 1 min, and 72°C for 30 s with 40 cycles, and then 5-min elongation at 72°C. Lane 1 is the 1-kb ladder [Bethesda Research Laboratories (BRL), Bethesda, Maryland] as size markers. The arrowhead indicates the expected 301-bp amplified TK product. (**B**) A 1.5% agarose gel showing primer-dependent amplification of mouse TK cDNA template pAMTK in the *E. coli* genomic background, with the arrow indicating the amplified TK product. PCR conditions were as in (A) with 2 μM dNTP. Lanes 1 to 3 used 10 ng of pAMTK as template in the absence of $T_{11}CA$ (lane 1), in the absence of Ltk3 (lane 2), and in the presence of both primers (lane 3). Lanes 4 and 5 were in the presence of both primers but with lysates of *E. coli* LE392 lacking plasmid pAMTK (lane 4) and LE392 harboring about 30 copies of the plasmid per cell (lane 5) as templates, respectively. Bacterial lysates were prepared from single colonies on agar plates as described (*16*). Lane 6 is the 1-kb ladder. (**C**) A 6% DNA sequencing gel showing [α-^{35}S]dATP–labeled PCR product. Samples were as in (B) except 0.5 μM [α-^{35}S]dATP (1200 Ci/mmol) was included from the first cycle in the PCR reactions and 6.5 μl of sample was analyzed. The arrowhead indicates the amplified TK product.

9 and 10 and 11 and 12) or the anchored oligo-dT primer (compare lanes 1 and 2 with 7 and 8; lanes 3 and 4 with 9 and 10; lanes 5 and 6 with 11 and 12), they exhibited totally different patterns, verifying the rationale on which this method is based. The additional two bases in the anchored oligo-(dT) primers provided marked specificity, recognizing different but numerically similar subpopulations of the total mRNA. In general, each lane exhibited about 50 to 100 bands for the arbitrary 10-mers (G-C content of 50%); arbitrary 9-mers were less polymorphic. Although shorter arbitrary primers should by statistics recognize more mRNA species than longer ones (Table 1), primers with nine or fewer bases were actually poorer primers, as shown here and also by others (*11*). Hence, under these PCR conditions with Taq DNA polymerase used at its optimal temperature of 72°C, the shorter primers may have too low melting temperatures (below 40°C) to bind efficiently. Arbitrary 10-mers in conjunction with anchored oligo(dT) primers, can amplify more bands than statistics allows, in theory acting like 6- or 7-mers. This result suggests that they may hybridize to the target mRNA sequences in a degenerate fashion during the first few PCR cycles; this is supported by alterations in the 5′ sequences of their products. This degeneracy is advantageous in revealing an optimal number of mRNA species per gel. In theory, about 10,000 6- to 7-mer sequences are possible. The chance of finding any one such sequence in 500 bases thus is 0.05. Therefore 20 arbitrary 10-mers (priming as 6- to 7-mers) should statistically cover all mRNA sequences upstream of the 12 possible anchored oligo(dT) primers.

The next step was to recover cDNA of an identified mRNA species from a dried DNA sequencing gel and reamplify it with PCR. To obtain a probe, a DNA band from the sequencing gel was electroeluted with Hoefer's gel eluter and ethanol precipitated to remove contaminants such as urea. The yield of DNA recovery was typically 50%, measured by radioactivity. The recovered DNA was reamplified in the presence of 20 μM dNTP to achieve optimal yield and specificity. The reamplified PCR products were often visible on an agarose gel after two consecutive 40-cycle PCR reactions (*14*). This suggests that these short primers can amplify DNA but at the cost of low priming efficiency; therefore, more cycles or even rounds of PCR are necessary to produce enough DNA to be seen on an agarose gel. Reamplifications of N1 and T1 were shown to be dependent on both primers, and products corresponded with their sizes on the original sequencing gel (*14*).

As an internal control for specific recovery, a band corresponding to TK from tumorigenic cells (Fig. 2B) was recovered and reamplified. The reamplified product was characterized by both its correct total length and Stu I digestion to give two fragments of the expected sizes, one being 124 bp and the other 177 bp (*14*).

The reamplified N1 was cloned into plasmid pCR1000 and sequenced. The nucleotide sequence clearly shows that the N1 fragment is flanked by the mRNA mapping primer sequences of Ltk3 at the 5′ end and $T_{11}CA$ at the 3′ end as expected (Fig. 4A). DNA sequence comparisons between the arbitrary primers in the recovered cDNAs such as N1 and their corresponding original cDNAs isolated from cDNA libraries show that there were two to three mismatches at the 5′ end of the arbitrary primers (*14*). Searching the GenBank and EMBL DNA databases revealed that the partial N1 cDNA clone shows 70% identity in nucleotide sequence to an expressed sequence tag (EST00839) recently isolated by random sequencing of a human brain cDNA library (*15*).

Northern (RNA) blot analysis with the N1 probe detected a single mRNA species of about 3.5 kb. N1 mRNA appeared to be present at low abundance in both gro and quiescent normal cells. The blot firms that N1 mRNA is present only ir normal A31 cells but not in the tun genic BPA31 cells (Fig. 4B). So far, a of four cDNA sequences have been ch terized. Of the three that were teste Northern blot analysis, two including turned out to be differentially expres The remaining one did not give any sig It is possible that its mRNA message ma too low to be detected by Northern bl

The differential display method scribed here is an alternative to subtrac or differential hybridization technique: can be used for three purposes. One i visualize mRNA compositions of cell displaying subsets of mRNAs as sl cDNA bands; samples run in parallel re differences in their mRNA patterns. Th useful in the same way as 2-D protein g for example, to see and identify alterat in gene expression. Second, these cDI can be quickly sequenced; thereby a tag each mRNA can be readily obtained compared with sequences in data ba

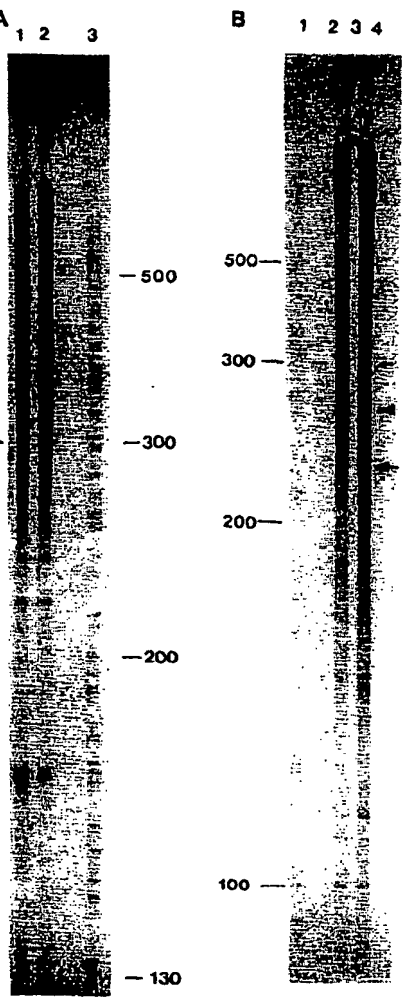

Fig. 2. Differential display using mRNA or t RNA. (**A**) Total RNA was isolated as descri (*17*). Two micrograms of the total RNA f A31 grown in Dulbecco's minimum esse medium (DME) with 10% bovine calf serum reaching 80% confluency (lane 1) or afte hours of 0.5% serum starvation (lane 2) w reverse-transcribed with 300 units of MM reverse transcriptase (BRL) in the presenc 2.5 μM of $T_{11}CA$ as primer and 20 μM dNTF 60 min at 35°C. After heat inactivation of reverse transcriptase at 95°C for 5 min, 2 μ the sample was added to 18 μl of PCR labe mix and amplified as in Fig. 2C. PCR proc (6.5 μl) was analyzed on a 6% DNA seque ing gel. Lane 3, single-stranded DNA s markers of single track (ddG) DNA sequenc of human TMP kinase cDNA (*18*) with prir 5′-AAAAGCTTCTGAAGTTGTGGGGT. The row indicates amplified TK mRNA only pres in the cycling cells (lane 1) but not in quiesc cells (lane 2). (**B**) Messenger RNA from 6 × BALB/c 3T3 mouse fibroblast cell lines A (normal) and BPA31 (tumorigenic) growing DME with 10% bovine calf serum and 10% C were isolated using the QuickPrep mRNA pu fication kit from Pharmacia–LKB Biochemi (Piscataway, New Jersey). The purified mRI (0.5 μg) either from A31 (lane 3) or BPA31 (la 4) were used as templates for the revei transcription and subsequent PCR amplific tion as described above. Lane 1, ssDNA s markers of single track (ddC) DNA sequenci of human TMP kinase as described. Lane 2, ng of pAMTK plasmid as template as exter control. The small arrow indicates amplified mRNA as an internal control. The arrowhe indicates an amplified mRNA species (N seen only in normal A31 cell but not in t tumorigenic cell BPA31. The large arrow in cates an amplified mRNA species (T1) fou only in the tumorigenic BPA31 cells.

Third, individual bands can readily be cloned and used as probes for Northern or Southern (DNA) blottings and to isolate genes from cDNA or genomic libraries.

Differential display has several technical advantages as compared to subtractive and differential hybridizations. It is much quicker; 2 months are required to isolate clones from cells by subtractive hybridization, which includes mRNA isolations, cDNA library constructions, subtraction, and screening by differential hybridization. With differential display the band patterns are obtained in 2 days and clones in 5 days. In addition, unlike subtractive hybridization, differential display allows simultaneous detections of both groups of differentially expressed genes (for example, candidate tumor suppressor genes and oncogenes). Most genes by statistics should be present in the patterns as single bands. Therefore, redundancy, underrepresentation of rare mRNAs, and false positive clones are minimized. In terms of sensitivity, because the method described here is PCR-based, only 1 μg of mRNA is required per 100 lanes, compared to 50 times as much or more for subtractive hybridization. A direct comparison of the number of cDNA bands amplified by a given set of primers with either a cDNA library or mRNA of the same cell type indicates that the new method is much more sensitive (*14*). This suggests possible underrepresentations of many genes during cDNA library constructions. Reproducibility from run to run of the method in displaying mRNA patterns with the same RNA sample is high (>95% bands are always seen) in comparison with the great variations in the kinds and numbers of genes isolated by each subtractive hybridization. The advantage of subtractive hybridization is its enrichment and focus on only the differentially expressed genes. Because of its simplicity based on PCR and a DNA sequencing gel, two of the most widely used molecular biological techniques, the differential display technique should find wide and rapid applications in studying cancer, heart disease, cell differentiation, and aging, among others.

These results demonstrate the potential of this technique to identify differentially expressed mRNAs and to clone their genes. It should make possible the detection of most of the mRNAs in a cell by use of multiple primer sets.

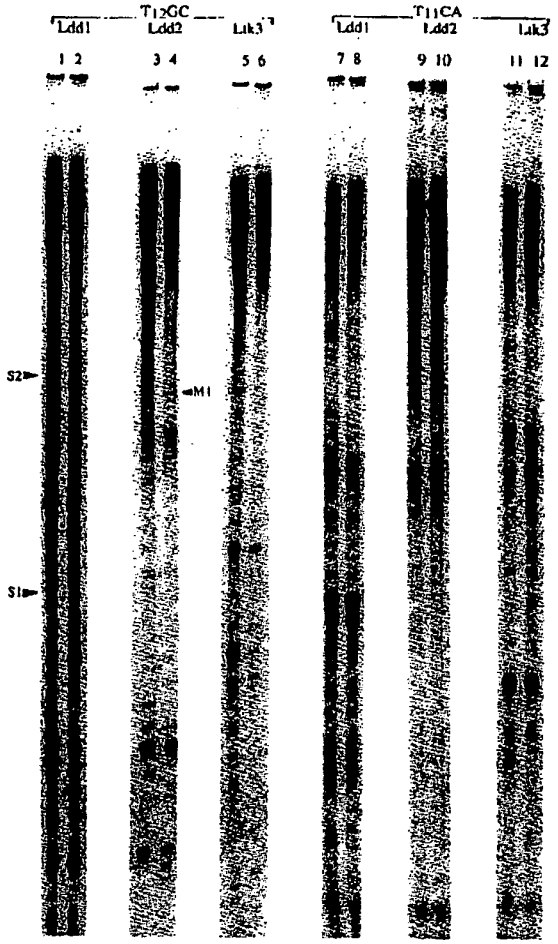

Fig. 3. Differential display of mRNAs from a normal versus a metastatic human breast cancer cell using multiple primer sets. Both normal (76N) and tumor (21MT-2) cells were cultured in D medium (*19*) until reaching about 70% confluency before the polyadenylated RNAs were extracted using the Quickprep mRNA purification kit from Pharmacia–LKB Biochemical. The purified mRNA (0.5 μg) was reverse transcribed with either $T_{11}CA$ or $T_{12}GC$. Six different combinations of primer sets made of two anchored oligo-dT primers ($T_{11}CA$: 5′-TTTTTTTTTTTCA-3′ and $T_{12}GC$: 5′-TTTTTTTTTTTGC-3′) and three short arbitrary primers (two are 10-mers, Ldd1: 5′-CTGATC-CATG-3′ and Ltk3: 5′-CTTGAT-TGCC-3′, and one is a 9-mer, Ldd2: 5′-CTGCTCTCA-3′) were used for the PCR reactions essentially as described, except the annealing temperature was at 40°C instead of 42°C. The odd-numbered lanes correspond to mRNA from normal cells whereas even-numbered lanes represent mRNA from the tumor cells. Several candidate cDNA tags that appear to be differentially expressed are marked by arrowheads.

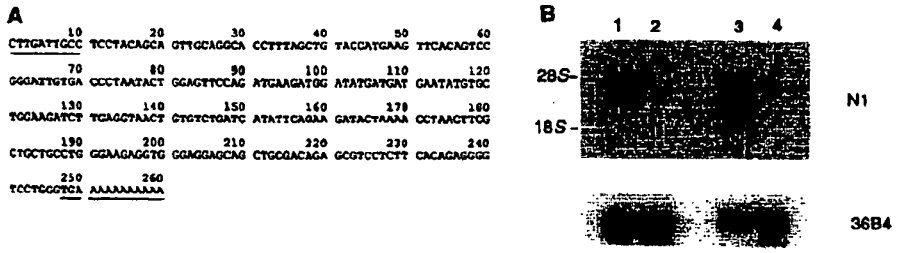

Fig. 4. (**A**) Nucleotide sequence of N1. Reamplified N1 fragment was cloned into pCR1000 to give pCR-N1 with the TA cloning system from Invitrogen (San Diego, California). Both strands of N1 were sequenced with the M13-40 and T7 promoter primers. Nucleotide sequences of flanking mRNA mapping primers are underlined. (**B**) Northern blot analysis of N1 mRNA in normal and tumorigenic mouse fibroblast cell lines. Cellular total RNA extraction and Northern blot were carried out as previously described (*20*). N1 was gel purified as an Eco RI–Hind III fragment from pCR-N1 and labeled with [α-^{32}P]dCTP to a specific activity of 1 × 10^9 cpm/μg of DNA with a random-prime DNA labeling kit from Boehringer Mannheim Biochemicals (Indianapolis, Indiana). A sample (20 μg) of total RNAs from either exponentially growing normal A31 cells (lane 1) and tumorigenic BPA31 cells (lane 2) or quiescent normal A31 cells (lane 3) and tumorigenic BPA31 cells (lane 4) obtained after 15 hours of serum starvation were analyzed. The blot was exposed for 72 hours with intensifying screens. As a loading control, the same blot was reprobed with 36B4 cDNA (*21*) and exposed for 16 hours without screen.

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